METHOD FOR INHIBITION OF VIRAL MORPHOGENESIS

Technical Field

The invention is directed to inhibiting viral morphogenesis and viral infection. In particular, it concerns effecting such inhibition by inhibiting the prenylation or post prenylation reactions of a viral protein.

Background Art

It has been shown that certain membrane-associated proteins require the addition of lipophilic residues in order to function properly. One family of such modifications is termed "prenylation" because the hydrophobic residue is derived from isoprenoid precursors. The prenyl residue is known to attach to the sulfhydryl group of a cysteine which has been shown in a number of membrane-associated proteins to be contained in a "CXXX" (SEQ ID NO: 1) box at the carboxy terminus of the substrate protein. In particular, one such membrane-associated protein has been shown to be the protein product of the ras oncogene. Summaries of these reactions conferring hydrophobic properties on membrane proteins, including prenylation, have appeared by Hoffman, M., Science (1991) 254:650-651, and by Gibbs, J.B., Cell (1991) 65:1-4.

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In addition, in many cases, prenylation is a
first step in a series of further reactions which modify
the carboxy terminus of prenylated proteins. These
prenylation initiated, or post-prenylation reactions
5   include carboxymethylation and proteolysis.

In the prenylation substrate proteins studied
to date, the CXXX (SEQ ID NO:1) box contains aliphatic residues in the
second and third positions and a leucine, serine,
methionine, cysteine or alanine in the terminal position.
10   Thus, in the CXXX (SEQ ID NO:1) boxes so far studied, the box itself is
relatively hydrophobic.

It has now been found that prenylation of a
viral protein is necessary for the morphogenesis of
hepatitis delta virus (HDV). This is the first
15   demonstration that viral proteins are subject to
prenylation. Furthermore, certain functional
consequences can be ascribed to prenylation. The viral
protein which is the target of prenylation, surprisingly,
contains a hydrophilic CXXX (SEQ ID NO:1) box of the sequence
20   Cys-Arg-Pro-Gln (SEQ ID NO:2). Prenylation, or prenylation-initiated
modification, of this relatively hydrophilic CXXX (SEQ ID NO:1) box and
corresponding CXXX (SEQ ID NO:1) boxes (hydrophilic or otherwise) or
other cysteine-containing sequences near the C-terminus
of proteins in other virions are suitable targets for
25   antiviral strategies.

These targets can now be seen to include, but
are not limited to, proteins of hepatitis A virus (HAV),
hepatitis C virus (HCV), herpes simplex virus (HSV),
cytomegalovirus (CMV), varicella-zoster virus (VZV),
30   influenza virus, plant viruses such as tobacco mosaic
satellite virus (TMSV) and barley stripe mosaic virus
(BSMV), the core antigen of hepatitis B virus (HBV) and
the nef gene product of human immunodeficiency virus-1
(HIV-1) -- especially since nef has been shown to play an
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important role in the development of AIDS. (Kesstler,
H.W. III, et al. Cell (1991) 65:651-662. Accordingly,
inhibition of the prenylation of these target proteins or
the post-prenylation reactions thereof is claimed to be
5   inhibitory to the progress of these infections.

Disclosure of the Invention
The invention provides methods to interfere
with viral morphogenesis, production, release or
10   uncoating both *in vitro* and *in vivo*. Agents which
interfere with the prenylation of, or the post-
prenylation reactions of, at least one viral protein are
provided to infected cells to halt the viral infection.
Such cells may be in culture or may be contained in an
15   animal or plant subject.
Thus, in one aspect, the invention is directed
to a method to inhibit viral morphogenesis, production,
release or uncoating which method comprises effectively
interfering with the prenylation of, or the post-
20   prenylation reactions of, at least one viral protein. In
another aspect, the invention is directed to an assay
method for screening candidate drugs for their ability to
inhibit prenylation. In a third aspect, the invention is
directed to a method for treating viral infection by
25   administering an agent effective to inhibit prenylation
of, or the post-prenylation reactions of, a viral
protein. In preferred embodiments, the viral protein is
the large delta antigen of the hepatitis D virus, core
antigen of HBV, or the nef protein of HIV.
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Brief Description of the Drawings
Figures 1A and 1B are photocopies of
immunoblots of proteins obtained by lysis of viral-
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infected cells expressing viral proteins and treated with tritiated mevalonate.

Figures 2A and 2B are photocopies of immunoblots of proteins derived from lysates of cells

5   containing wild type or mutant viral proteins and labeled with tritiated proline or mevalonate.

Figures 3A, 3B, 3C and 3D are photocopies of immunoblots of various cell supernatants containing viral proteins.

10   Figure 4 is a diagrammatic representation of the progress of HDV morphogenesis.

Modes of Carrying Out the Invention

Hepatitis delta virus (HDV) infections cause

15   both acute and chronic liver disease and can be fatal (1, 2). This RNA virus contains a 1.7 kb single-stranded circular genome and delta antigen, the only known HDV-encoded protein. These elements are encapsulated by a lipid envelope in which hepatitis B virus surface

20   antigens are embedded (3), which explains why HDV infections occur only in the presence of an accompanying HBV infection (4, 5). Two isoforms of delta antigen exist in infected livers and serum (6, 7). This heterogeneity arises from a unidirectional mutation at a

25   single nucleotide in the termination codon for delta antigen (codon 196: UAG → UGG), which occurs during replication (8). Thus, although small delta antigen is 195 amino acids long, large delta antigen is identical in sequence except that it contains an additional 19 amino

30   acids at its COOH terminus. Although both forms of delta antigen contain the same RNA genome binding domain (9), they have dramatically different effects on genome replication. The small form is required for replication,

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whereas the large form is a potent trans-dominant inhibitor (10, 11).

The last four amino acids of large delta antigen are Cys-Arg-Pro-Gln-COOH. (SEQ ID NO:2) This COOH-terminal configuration, termed a CXXX box (SEQ ID NO:1) (where C is cysteine and X is any amino acid), has been implicated as a substrate for prenyltransferases that add to the cysteine 15 (farnesyl) or 20 (geranylgeranyl) carbon moieties derived from mevalonic acid (12-14). The resulting hydrophobic modification may aid in membrane association of the derivatized protein, as suggested for p21 Ras (15, 16) and lamin B (12, 17). We have now demonstrated that large delta antigen is similarly modified.

Other virions also contain suitable target sequences for prenylation. These sequences are near the carboxy terminus of the viral protein targeted, and may be in the form of CXXX boxes (SEQ ID NO:1), but the cysteine may also be closer to the C-terminus, including a position as the C-terminal amino acid, as is the case of the core antigen of hepatitis B virus (HBV) and the nef gene product of HIV-1.

To determine whether large delta antigen is a substrate for prenylation, we labeled three cell lines, SAG, LAG, and GP4F, with [^{3}H]mevalonic acid. GP4F cells are a derivative of NIH 3T3 cells (18). SAG (19) and LAG (20) cells are derivatives of GP4F cells that stably express the small and large delta antigens, respectively.

Labeled cell lysates were analyzed on immunoblots (Fig. 1A) to detect steady-state amounts of small and large delta antigen. The lysates were also subjected to immunoprecipitation with an antibody to the delta antigens (anti-delta), SDS polyacrylamide gel electrophoresis (SDS-PAGE), and fluorography (Fig. 1B).

In more detail, referring to Fig. 1, large
delta antigen is shown to be prenylated in cultured
cells. The cell lines SAG (*19*) (lane 1), LAG (*20*) (lane
2), and GP4F (*18*) (lane 3) were grown overnight in
5   Lovastatin (25 μM) and (R,S)-[5-^{3}H]mevalonate (140 mM))
(*30*), and lysed in RIPA buffer [50 mM Tris (pH 7.5), 150
mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS)
(*20*). (A) Aliquots were subjected to immunoblot analysis
(*11*). The blot was treated with serum from an HDV-
10   infected patient that contained antibody to delta antigen
(α-δAg) and horseradish peroxidase-conjugated rabbit
antibody to human immunoglobulin G (IgG) (Promega),
followed by chemiluminescence (Amersham) development.
(B) Immunoprecipitates (with α-δAg) from cell extracts
15   were subjected to SDS-PAGE and fluorography. As shown in
Figure 1, S, small delta antigen, L, large delta antigen.
Molecular size markers are shown at the left (in
kilodaltons).

Thus, the large, but not the small, antigen was
20   labeled with [^{3}H]mevalonic acid, suggesting that large
delta antigen undergoes prenylation in cultured cells.

We obtained similar results using *in vitro*
translation reactions (13) performed in the presence of
[^{3}H]proline or [^{3}H]mevalonate (Fig. 2). Fig. 2. also
25   shows mutation of Cys^{211} of large delta antigen to Ser
and loss of prenylation. In vitro translation reactions
were performed with rabbit reticulocyte lysates (Promega)
in the presence of either (A) L-[2,3,4,5-^{3}H]proline (19
μM) (94 Ci/mmol, Amersham) or (B), [^{3}H]mevalonate (200
30   μM) (*30*). For (A) and (B), translation reactions
contained small delta antigen mRNA (lane 1); large delta
antigen mRNA (lane 2); water (lane 3); or large delta
antigen (Cys^{211} → Ser) (*20*) mRNA (lane 4). A portion 20
μl) of each reaction was added to 1 ml of RIPA buffer,
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immunoprecipitated with α-δAg, and analyzed as described
(Fig. 1).

Both the small and the large antigens were
labeled with [^{3}H]proline (Fig. 2A), whereas only the
5   large isoform was labeled with [^{3}H]mevalonate (Fig. 2B).
To determine whether modification by [^{3}H]mevalonate was
dependent on the presence of Cys^{211} in the terminal CXXX
(SEQ ID NO: 1)
box, we constructed a mutant that contains a serine at
this position (20). Cys^{211} is the only cysteine in large
10   delta antigen. Mutating Cys^{211} to Ser did not interfere
with the synthesis of large delta antigen (Fig. 2A) but
abolished its modification by [^{3}H]mevalonate (Fig. 2B).

The specific type of mevalonate modification of
large delta antigen appears to be geranylgeranyl rather
15   than farnesyl (21). Although the first described CXXX
(SEQ ID NO: 1)
boxes contained aliphatic residues at the first and
second positions after Cys, other types of amino acids
can be found in prenylation sites (13, 14). It is not
clear whether the COOH-terminal sequence
(SEQ ID NO: 2)
20   Cys-Arg-Pro-Gln-COOH, which differs from that of
(SEQ ID NO: 1)
previously described CXXX boxes, implies the existence of
a novel prenylation enzyme or whether it reflects a
broader substrate specificity of known
prenyltransferases.

25   For HDV particle formation, delta antigen and
associated genomes are presumably targeted to cell
membranes that contain HBV envelope proteins. We
hypothesized that prenylation of large delta antigen
could be involved in this process. We first examined
30   whether large delta antigen was sufficient for HDV-like
particle formation. HBV surface antigen (HBsAg) was
expressed transiently in COS-7 cells together with small
or large delta antigen. Virus-like particles consisting
of delta antigen packaged into HBsAg-containing envelopes
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were analyzed by immunoprecipitation of clarified media supernatants with an antibody to HBsAg (anti-HBs).

Fig. 3 shows particle formation with large delta antigen and HBsAg parts. For panels (A) and (B), COS-7 cells were transiently transfected with the following plasmids: SV24H, which expresses HBV surface antigen (31), and SVLAg, which expresses small delta antigen (19) (lane 1); SV24H and SVL-large, which expresses large delta antigen (20) (lane 2); and calcium phosphate precipitate without DNA (lane 3). In (C) and (D), COS-7 cells were transfected with SV24H and SVL-large (lane 4); SV24H and SVL-large (Ser^{211}) (20) (lane 5); and calcium phosphate precipitate without DNA (lane 6). For (A) and (C), 48 hours after transfection, HBsAg-containing particles were immunoprecipitated from 2-ml aliquots of clarified media supernatants with anti-HBs (31) and subjected to immunoblot (with α-δAg) and chemiluminescence analyses as described (Fig. 1). For (B) and (D), the transfected cells were harvested in cell lysis buffer [50 mM Tris (pH 8.8), 2% SDS] with protease inhibitors (20), and aliquots subjected to protein immunoblot and chemiluminescence analyses. Molecular size markers are shown at the left (in kD).

The presence of delta antigen in the immunoprecipitates was assayed by immunoblot analysis (Fig. 3A). Although both small and large antigens were synthesized in the transfected cells (Fig. 3B), only the large isoform was incorporated into secreted HBsAg-containing particles (Fig. 3A). Similar selective packaging has been observed (22).

We then examined the function of mevalonate modification in this particle formation. One explanation for the preferred packaging of large delta antigen is

-9-   (SEQ ID NO:1)

that the small antigen lacks the CXXX box and therefore
cannot undergo modification. The Cys^{211} → Ser mutant of
large delta antigen should behave like small delta
antigen and not be packaged. This was indeed found to be

5   the case. Whereas both wild-type and Ser^{211} mutant large
antigens were synthesized in transfected cells (Fig. 3D),
only the wild-type form was packaged into particles
(Fig. 3C). Thus, the mutated form of large delta antigen
is not prenylated and cannot form particles with HBsAg.

10   Our results suggest that prenylation of large
delta antigen is required for the formation and release
of particles containing delta antigen and HBV surface
antigens. The requirement of a prenylation site for
productive viral infection is further suggested by other

15 mutations of the CXXX box (23) *(SEQ ID NO:1)* and by the conservation of
Cys^{211} and a CXXX box motif *(SEQ ID NO:1)* among all sequenced HDV
isolates (24).

           The ability of large, but not small, delta
antigen to be prenylated and packaged into virus

20 particles further highlights the significance of the
mutation-induced heterogeneity at the termination codon
of the small delta antigen. During HDV replication, S
genomes (encoding the small antigen) mutate to L genomes
(encoding the large antigen). At least two effects

25 attributable to this mutation can be distinguished (see
Fig. 4). Fig. 4 shows the regulatory switch of S genomes
to L genomes. During replication, S genomes encoding the
small delta antigen mutate to L genomes, which encode the
large delta antigen. This single base mutation has two

30 effects on the COOH-terminus of delta antigen. The first
is to change the nature of the COOH-terminal amino acid;
Pro (P), which enhances genome replication (20), is
replaced by Gln (Q), resulting in inhibition of genome
replication. The second effect is the creation of a

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(SEQ ID NO: 2)

A   target prenylation site (CRPQ), C, cysteine; R, arginine; P, proline; Q, glutamine.

        Thus, the first effect is the conversion of an enhancer of genome replication (small delta antigen) into
5   a potent trans-dominant inhibitor (large delta antigen) (10, 11). This dramatic difference in function appears to be determined solely by the nature of the COOH-terminal amino acid with proline being sufficient to confer enhancer activity (11, 25). The second effect is
A 10 the addition of a CXXX box *(SEQ ID NO:1)* to delta antigen, which allows the protein to be prenylated and presumably promotes its incorporation into HBsAg-containing particles. The combined effects of the switch from production of small to large delta antigen thus appear to have two roles: to
15   suppress further genome replication and to promote the onset of packaging and virion morphogenesis.

        Our results suggest prenylation as a new target for anti-HDV therapy and for antiviral therapy with respect to other viruses with prenylated proteins. Such
20   therapy is directed at inhibiting virion morphogenesis, production, release and uncoating (functionally the reverse reaction of virion morphogenesis). In light of the increasingly apparent degeneracy of the four C-terminal amino acids required to function as a
25   prenylation substrate, a cysteine located at any of these C-terminal positions is also considered to identify a potential target of antiprenylation therapy.

        Several strategies designed to interfere with the prenylation stage of the HDV life cycle may be
30   considered, including drugs that inhibit enzymes along
A   the prenylation pathway, and CXXX box *(SEQ ID NO:1)* analogs. Both therapies have been considered for the inhibition of ras-mediated oncogenic transformation (26). Tetrapeptides
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35   that correspond to the CXXX box *(SEQ ID NO:1)* of p21 Ha-Ras inhibit

prenylation of p21 Ha-Ras *in vitro* (27). Finally, the
dual function of large delta antigen in the HDV life
cycle suggests a further refinement of a proposed (11)
defective interfering particle- (DIP) (28) like therapy

5   aimed at cells infected with actively replicating S
genomes. Because L genomes require a source of small
delta antigen for replication (19, 29) but, once
replicated, produce a potent trans-dominant inhibitor of
further replication, a therapeutically administered L

10   genome DIP could be specific for infected cells, as well
as possess an inherent shut-off mechanism (11). If the L
genome also contained the Cys^{211} to Ser mutation, it
could encode a delta antigen that not only inhibits
replication but also affects packaging.

15   Accordingly, new approaches to antiviral
therapy and inhibition of viral morphogenesis focus on
inhibition of the prenylation of, or post-prenylation
reactions of, at least one viral protein. This may be
effected by contacting cells infected with the target

20   virus with an effective amount of an agent which inhibits
the prenylation of, or post-prenylation reactions of, at
least one viral protein. Such agents include inhibitors
of formation of the prenyl groups which are derivative of
the mevalonate synthesis pathway. Other agents include

25   decoys for the target sequence for prenylation, including
small peptides, including tetrapeptides and other
compounds which mimic the surroundings of the cysteine
residue to be prenylated. For example, Reiss, Y., et al.
<u>Cell</u> (1990) 62:81-88 report prenylation inhibition by

30   C-A-A-X (SEQ ID NO:3) tetrapeptides. As set forth above, the cysteine
residue to be prenylated is generally found at the
carboxy terminus of the target protein; although the most
common target sequence involves a CXXX (SEQ ID NO:1) box, cysteines
positioned closer to the C-terminus may also be targeted;

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thus, the relevant peptides may include those of the form XCXX, XXCX, and XXXC. (SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6) Other agents include derivatives and mimics of prenyl groups themselves. Other suitable agents include inhibitors of the prenyltransferase

5 enzymes and of enzymes that catalyze post-prenylation reactions.

Assay of Candidate Inhibitors

    The present invention also provides a method to

10 screen candidate drugs as prenylation inhibitors by taking advantage of the requirement for prenylation in order to effect secretion of certain prenylated proteins. For those proteins for which secretion requires prenylation, the assay can be conducted in a direct and

15 simple manner. Cells that secrete, or that have been modified to secrete, a first protein whose secretion is dependent on prenylation are used as the experimental cells. A second protein which does not depend on prenylation for secretion is used as a control. This

20 control protein may be secreted by the same or different host cells as the first protein. The candidate drug is applied to cells that secrete both proteins, or to matched sets of cells that secrete each. Secretion can readily be assessed by assaying the cell supernatants for

25 the presence or absence of the first and second secreted proteins using, for example, routine ELISA assays. Successful candidate drugs will not inhibit the secretion of the control protein, but will inhibit the secretion of the protein in the test sample wherein prenylation is

30 required for secretion.

    The large delta antigen of HDV is a viral protein for which prenylation is a prerequisite for secretion. Thus, this protein forms, itself a key part of a useful test system for the assay. Cells that are

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modified to secrete a protein for which prenylation is not required can be used as controls. If large delta antigen is used as the test protein, it is advantageous to use HBsAg as the control protein in the same cell since HBsAg is also required for secretion of delta antigen.

The foregoing assay, of course, requires that the inhibitor interfere with the prenylation system for large delta antigen or for any other prenylation-controlled secreted protein used in the assay. A range of prenyl transferases and prenyl groups is known to apply to various proteins for which prenylation inhibitors are required or sought. Some of these proteins are not secreted, whether they are prenylated or not; one such example is the protein product of the ras oncogene.

Nevertheless, the assay system described can be employed to screen for inhibitors of prenylation in these nonsecreted proteins by providing the target "CXXX" box (SEQ ID NO:1) characteristic of the nonsecreted protein in place of the corresponding "CXXX" box (SEQ ID NO:1) of the secreted one. The resulting chimeric protein will exhibit the prenylation characteristics of the imported "CXXX" box (SEQ ID NO:1) characteristic of the nonsecreted protein, but retain the ability of the host secreted protein to be passed to the supernatant for assay. Thus, the range of target proteins for which prenylation inhibitors are sought by use of the assay can be expanded to nonsecreted proteins.

The presence of a control system which provides secreted protein not dependent on prenylation is critical. The presence of this control allows candidate inhibitors which merely are toxic to the cells, or which inhibit secretion in general, to be discarded. Prenylation inhibitors identified by one of the

variations of the above described assay are expected to find use not only in the inhibition of viruses, but also in other processes or disease states -- including but not limited to cancer -- in which a prenylated protein is 5 found to be involved.

Evidently, prenylation of viral proteins is a prerequisite for additional post-prenylation reactions of the proteins such as proteolysis and carboxymethylation. The essential sequence of steps can be interfered with at 10 the most convenient point for the viral protein in question.

Administration of the Inhibitors

Additional viral proteins subject to 15 prenylation can be obtained by screening amino acid sequence data banks for viral proteins which contain a (SEQ ID NO: 1) "CXXX" box at the C-terminus. An illustrative list of such proteins includes, for example, specific proteins of HAV, HCV, HSV, CMV, VZV, influenza virus, plant viruses 20 such as tobacco mosaic satellite virus and barley stripe mosaic virus, core antigen of hepatitis B virus and the nef gene product of HIV I, as set forth above. These candidates for suitable prenylation targets can be validated in a manner similar to that described above by 25 providing labeled mevalonic acid to cells infected with or containing the appropriate viruses or viral gene products, and assessing the prenylation status of the viral proteins obtained using incorporation of label as the criterion. Furthermore, the role of prenylation in 30 the morphogenesis of the respective virions, and its suitability as a target for anti-viral therapy, can also be validated in a manner similar to that described above.

If viral morphogenesis, production, release or uncoating are to be inhibited in culture, suitable host 35

cells are used to culture the virus, and the agents used in inhibiting prenylation or post prenylation reactions added to the medium. If the infected cells are contained in an animal subject, such as a mammalian subject or in

5 particular a human or other primate subject, the agent used for the prenylation inhibition is generally introduced as a pharmaceutical formulation. - Suitable formulations depending on the nature of the agent chosen may be found in Remington's Pharmaceutical Sciences,

10 latest edition, Mack Publishing Co., Easton, PA. The routes of administration include standard such routes, including administration by injection, oral administration, and transmucosal and transdermal administration. The choice of formulation will depend on

15 the route of administration as well as the agent chosen. Suitable mixtures of agents can also be used as active ingredients. For administration to plants, formulations which are capable of conducting the active ingredients into plant cells are used as carriers.

20 The following references are listed according to the number which refers to them in the body of the specification:

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